BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

[CANCER RESEARCH 54, 5745–5751, November 15, 1994]

Advances in Brief

Long-Term Survival of Rats Harboring Brain Neoplasms Treated with Ganciclovir and a Herpes Simplex Virus Vector That Retains an Intact Thymidine Kinase Gene[1]

Efstathios J. Boviatsis, John S. Park, Miguel Sena-Esteves, Christof M. Kramm, Maureen Chase, James T. Efird, Ming X. Wei, Xandra O. Breakefield, and E. Antonio Chiocca[2]

Neurosurgery Service [E. J. B., E. A. C.] and Molecular Neurogenetics Laboratory, Department of Neurology [E. J. B., J. S. P., M. S. E., M. C., C. M. K., M. X. W., X. O. B., E. A. C.] and Biostatistics Unit, Department of Radiation Oncology [J. T. E.], Massachusetts General Hospital, and Program in Neurosciences [X. O. B.], Harvard Medical School, Boston, Massachusetts 02114

Abstract

Survival of rats harboring cerebral 9L gliosarcomas can be significantly extended by an intratumoral inoculation with a herpes simplex virus vector, designated as hrR3. This vector, which bears the *lacZ* reporter gene, is defective in the gene encoding ribonucleotide reductase, allowing for replication in dividing tumor cells but not in postmitotic neural cells. It also possesses an intact viral thymidine kinase (*TK*) gene, which confers chemosensitivity to ganciclovir. In this study, the ability of ganciclovir to potentiate the antitumor effect of hrR3 was evaluated. In culture, there was a 23% decrease in the growth of 9L cells treated with hrR3 plus ganciclovir compared to hrR3 alone ($P < 0.01$). The combination of hrR3 plus ganciclovir led to the long-term survival of 48% of rats harboring intracerebral 9L gliosarcomas compared to 20% survival in the hrR3 group ($P < 0.05$). Ganciclovir treatment had no effect on the growth of tumor cells *in vitro* or *in vivo* when a herpes simplex virus vector with a defective *TK* gene was used. Immunocytochemistry confirmed selective expression of the *TK* gene in cells within the tumor. These findings indicate that the *TK* gene can potentiate the antitumor effect of the hrR3 herpes simplex virus vector and provide the basis for placing additional therapeutic genes in the genome of hrR3.

Introduction

The insertion of foreign genes into tumor cells holds great promise as a novel therapeutic approach in neuro-oncology. The type of vector used for gene delivery is an important parameter that could affect antitumor efficacy (1). Vectors based on HSV[3] mutants with genetic defects in enzymes needed for nucleic acid metabolism possess the dual properties of: (*a*) tumor cytotoxicity, since they selectively replicate in dividing cells (2, 3); and (*b*) foreign gene transfer, since they can mediate the efficient expression of a reporter gene in tumor cells in the brain in a relatively selective fashion (1, 4). We have shown previously that an HSV mutant, defective in the enzyme ribonucleotide reductase (designated as hrR3; Ref. 5) preferentially expresses the *lacZ* gene from *Escherichia coli* in tumor cells as compared to normal cells in the brains of rats (4). The cytotoxic action of the replicating HSV vector toward tumor cells was itself sufficient to support long-term survival (greater than 70 days) of approximately 20% of rats treated intratumorally with hrR3. Of note, this mutant maintains an intact viral *TK* gene, allowing for potential chemosensitivity of tumor cells to the prodrug, ganciclovir (6). In this report, we show that death of 9L gliosarcoma cells can be enhanced *in vitro* by the combination of hrR3 and ganciclovir. The long-term survival of rats with intracerebral 9L tumors treated with hrR3 followed by ganciclovir was improved significantly compared to that of rats treated with hrR3 alone. Immunocytochemistry for *TK* showed selective expression of the enzyme in cells within the tumor. These results indicate that the tumoricidal action of HSV vectors, defective in enzymes needed in nucleic acid metabolism, can be enhanced through the inclusion of drug-susceptibility genes.

Materials and Methods

Cell Lines and HSV Vectors. The rat 9L gliosarcoma cell line was obtained from the Brain Tumor Research Center (University of California at San Francisco; Ref. 7). Cells were grown in DMEM with 10% FCS and PS (GIBCO-BRL, Gaithersburg, MD) at 37°C in 5% carbon dioxide. The terms HSV vector, HSV mutant, and HSV recombinant are used interchangeably in this report. The RH105 HSV vector, whose genotype is TK(−)LacZ(+), was obtained from Drs. D. Ho and E. Mocarski (Stanford University Medical Center; Ref. 8). The hRR3 HSV vector, whose genotype is RR(−)LacZ(+), was obtained from Dr. S. Weller (University of Connecticut Medical School; Ref. 5). The above HSV vectors possess insertions of the *E. coli lacZ* gene into the *TK* and *RR* gene loci, respectively. Expression of the *lacZ* gene is driven by the ICP4 and ICP6 immediate-early viral promoters in RH105 and hrR3, respectively. The above HSV vectors were stored in stocks at −80°C prior to their use. They were passaged on African green monkey (Vero) cells, and viral titers were obtained by plaque-formation assays on Vero cell monolayers. Procedures involving viruses were in accordance with guidelines issued by the Harvard Office of Biological Safety.

Cell Culture Studies. 9L gliosarcoma cells were plated at a density of 3×10^5 cells per p60 dish (Fisher, Pittsburgh, MD) in 5 ml of DMEM/FCS/PS. The following day, three random dishes were trypsinized and counted using a Coulter counter. Based on an average cell number of 4.9×10^5 cells per dish, HSV vectors, RH105 or hrR3, were thus added to achieve a MOI of 0.01. This MOI was selected based on previous studies showing that it resulted in 50% inhibition of 9L cell growth within 6 days (4). Experiments were performed in triplicate. Dishes were incubated at 37°C and monitored daily using a phase-contrast microscope. After three days, ganciclovir (Cytovene; Syntex) was added to each dish at a concentration of 1 μg/ml. Control dishes were treated with saline. Two days after the addition of the drug, dead cells were removed by repetitive washing with Hanks' buffered saline (GIBCO-BRL); cells that remained attached to the dish were trypsinized and counted in a Coulter counter. Three dishes of 9L gliosarcoma cells that had not been infected with the HSV vectors were assayed in parallel. No cell rounding or lifting was evident in the control dishes over the total incubation period of 6 days.

Animal Studies. Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Animal Care. Viral inoculation and care of animals harboring viruses were performed

Received 8/5/94; accepted 10/6/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the NIH National Institutes of Neurologic Disorders and Stroke NS 24279-08 (to E. A. C. and X. O. B.). E. J. B. is a Fellow of the Alexandros Onassis Foundation. C. M. K. is a fellow of the Deutsche Krebshilfe.

[2] To whom requests for reprints should be addressed, at Molecular Neurogenetics Laboratory, 6th Floor, Massachusetts General Hospital-East Building, Charlestown, MA 02129.

[3] The abbreviations used are: HSV, herpes simplex virus; TK, thymidine kinase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PS, penicillin/streptomycin; MOI, multiplicity of infection; PBS, phosphate-buffered saline.

in approved viral vector rooms. Male Fischer 344 CD rats (200–300 g) were obtained from Taconic Laboratories. They were anesthetized through an i.p. injection of 0.7 ml of 0.63% sodium chloride, 10% ethanol, and 20% sodium pentobarbital (from a 64.8 mg/ml solution available from Anthony Products, Arcadia, CA). After immobilizing the rats in a stereotactic apparatus (Kopf), a linear skin incision was performed on top of the skull. A burr hole was drilled in the skull with a high-speed drill approximately 1 mm in front and 2 mm lateral to the bregma on the right side. After incising the dura with a sterile needle and obtaining hemostasis, 9L gliosarcoma cells (4×10^4 in a volume of 5 μl of DMEM) were injected with a Hamilton syringe at a depth of 3.5 mm from the dura over the course of 5 min. After retracting the needle over the course of 2 min, the skull was washed with 0.9% sodium chloride, and bone-wax (Ethicon) was used to occlude the burr hole. Five days later, animals were reanesthetized and replaced onto the stereotactic apparatus. The HSV vectors defective in *TK* or *RR* (RH105 or hRR3, respectively) were inoculated in DMEM/FCS/PS (2×10^7 plaque-forming units in 10 μl). Control animals received a stereotactic injection of vehicle alone. Stereotactic injections were carried out for each vector over the space of 10 min with needle retraction carried out over 5 min. i.p. injections of ganciclovir (7.5 mg/kg every 12 h) or saline were started 7 days after HSV vector inoculation (12 days after tumor implantation) and were carried out for 7 days.

For histopathological analysis, rats treated with hrR3 plus saline or hrR3 plus ganciclovir were sacrificed after 7 days of ganciclovir treatment by *in vivo* perfusion, and their brains were histologically analyzed by cresyl violet staining as described (1, 4).

The double staining procedure of tissue sections for *lacZ* gene expression and for *TK* antigen immunocytochemical detection was carried out by first staining with Xgal (Sigma Chemical Co.) as described (1, 4) and then by placing sections 10 min in 1% hydrogen peroxide in methanol, followed by extensive washing in 10 mM sodium phosphate and 0.3% Triton-X in 0.9% sodium chloride, pH = 7.3 (PBS). The primary antibody was obtained from Dr. William Summers, Yale University School of Medicine. It consists of serum raised in rabbits against highly purified recombinant HSV-*TK*. It was added onto sections overnight at a 1:1000 dilution in 1% bovine serum albumin-10% normal goat serum in PBS. After washing, detection of antibody-antigen complexes was performed by first using a commercially available kit (Vectastain ABC kit; peroxidase-conjugated anti-rabbit IgG; Vector Laboratories) and then, after washing in PBS (without Triton-X), by final incubation in a solution containing 50 mg of 3,3′-diaminobenzidine (Sigma) and 0.006% hydrogen peroxide in 100 ml of PBS (without Triton). After counterstaining 5 min in hematoxylin, sections were dehydrated first in ethanol and then in xylene before mounting on slides.

Results

***In Vitro* Studies.** We have shown previously that approximately 50% 9L gliosarcoma cell death was achieved with hrR3 and RH105 at an MOI = 0.01 in 6 days (4). Here we evaluated whether addition of ganciclovir would enhance the cytotoxicity of hrR3, the HSV vector that retains an intact *TK* gene. Table 1 shows that addition of RH105 or hrR3 at a MOI of 0.01 decreased the number of 9L cells by 63 and 38%, respectively, 5 days after infection. Addition of ganciclovir to control 9L cells or 9L cells infected with RH105 (the HSV mutant defective in *TK*) did not affect the number of 9L cells. However, addition of ganciclovir to 9L cells that had been infected with hrR3 (the HSV mutant that retains an intact *TK* gene) significantly in-

Table 1 *9L gliosarcoma cell proliferation after treatment with the RH105 or the hrR3 HSV vectors with or without ganciclovir*

	Control	RH105	hrR3
− Ganciclovir	10.25[a] (3.63)[b]	3.81 (1.13)	6.41 (2.03)
+ Ganciclovir	9.99 (4.23)	4.15 (2.28)	4.91[c] (2.63)

[a] Values represent average number of 9L gliosarcoma cells ($\times 10^6$) 5 days after plating on a p60 dish. All experiments were run in triplicate.
[b] Values in parentheses represent SE.
[c] The difference between the values in the hrR3 column (− *versus* + ganciclovir) was statistically significant ($P < 0.05$; Student's t test). The differences between the values in the control column or in the RH105 column was not significant ($P > 0.1$).

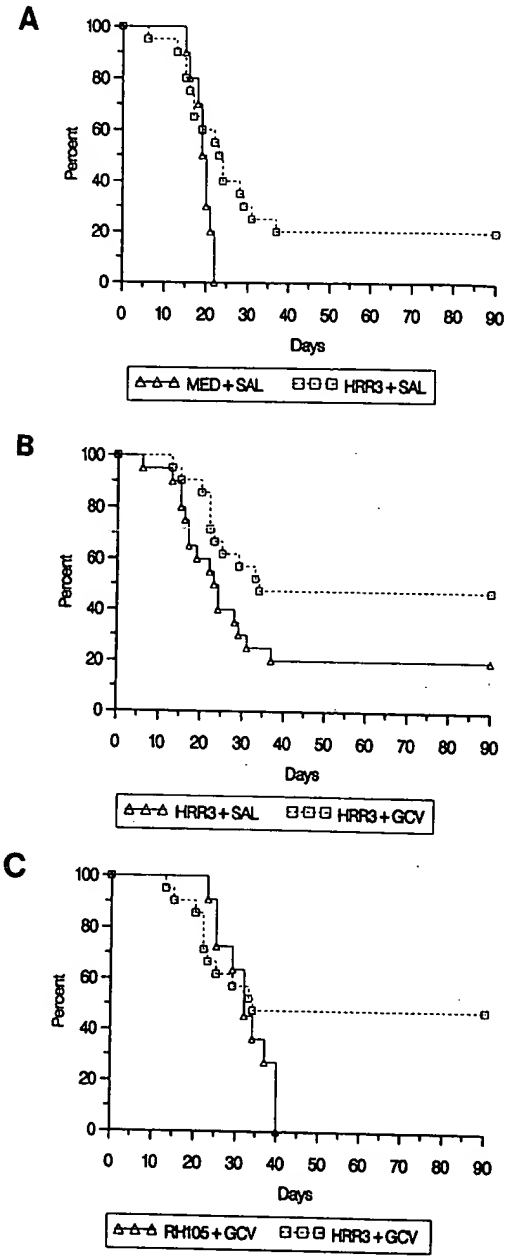


Fig. 1. *A*, Kaplan-Meier survival analysis for rats with brain tumors treated with the hrR3 HSV vector. Five days after implanting 40,000 9L gliosarcoma cells in the frontal lobe of Fischer 344 rats, animals were treated with a stereotactic inoculation of hrR3 (2×10^7 plaque-forming units in 10 μl; $n = 20$) or vehicle (*MED*; $n = 10$). Seven days later, daily i.p. injections of saline were administered for 7 days in order to compare with rats treated with ganciclovir (see next panels). Log-rank statistical analysis revealed that the two survival curves were different ($P < 0.05$). Portions of this experiment were published in a previous report (4). *B*, Kaplan-Meier survival analysis for rats with brain tumors treated with the hrR3 HSV vector in the the presence or absence of ganciclovir. The group treated with hrR3 and saline (see panel *A*) was compared to a group treated with hrR3 and ganciclovir ($n = 21$). Ganciclovir (7.5 mg/kg given twice per day) was administered for a total of 7 days after inoculation of hrR3. Log-rank statistics revealed that the two survival curves were different ($P < 0.05$). *C*, Kaplan-Meier survival analysis for rats with brain tumors treated with the hrR3 HSV vector plus ganciclovir or the RH105 HSV vector (that has a defective *TK* gene) plus ganciclovir. The group treated with hrR3 and ganciclovir was compared to a group treated with RH105 (an HSV vector that has a defective *TK* gene) and ganciclovir (same dosing schedule as in panel *B*). A statistical comparison of the means and SE between the two groups revealed a significant difference ($P < 0.01$; Student's t test). For panels *A-C*, the beginning of the time axis marks the day that animals were injected with tumor cells.

creased cytotoxicity as compared to hrR3 infection without ganciclovir ($P < 0.05$; Student's t test). These results indicated that ganciclovir potentiated the antitumor effect of hrR3.

Survival Comparison between hrR3 and Saline. The antitumor activity of hrR3 was shown previously to result in: (*a*) long-term survival of rats harboring 9L gliosarcomas in their frontal lobes; and (*b*) histopathological evidence of extensive tumor necrosis (4). The survival findings were reevaluated using Kaplan-Meier survival analysis (9). Tumors were implanted in the right frontal lobes of rats and, 5 days later, a stereotactic injection of either hrR3 ($n = 20$) or vehicle ($n = 10$; Fig. 1*A*, *MED*) was performed. Seven days later, i.p. injections of saline were carried out for a total of 7 days. Fig. 1*A* shows the Kaplan-Meier survival curves for both groups. There were no survivors in the vehicle-treated group, while 20% of animals treated with hrR3 survived longer than 90 days and were still alive at the time of manuscript submission (5 months). Log-rank statistical analysis showed that this survival was significant ($P < 0.05$). Therefore, this indicated that the inherent cytotoxic action of hrR3 could result in long-lasting and complete regression of brain tumors in 20% of treated rats.

Survival Comparison between hrR3 and hrR3 Plus Ganciclovir. The hrR3-HSV vector possesses an intact *TK* gene which confers ganciclovir sensitivity to 9L cells in culture. We thus determined whether the cytotoxic effect of hrR3 could be potentiated by ganciclovir. Five days after the implantation of 9L tumors in the frontal lobes of rats, hrR3 was stereotactically inoculated into the tumor. Seven days later, rats received daily i.p. injections of saline ($n = 20$) or ganciclovir ($n = 21$) for a 7-day period. Kaplan-Meier survival analyses are shown in Fig. 1*B*. Ten of 21 rats (48%) treated with hrR3 plus ganciclovir survived longer than 90 days and were still alive at the time of manuscript submission (5 months), as compared to the 4 of 20 animals (20%) treated with hrR3 alone. Log-rank statistical analysis revealed that this difference in survival was significant ($P < 0.05$). This indicated that the antitumor effect of hrR3 was significantly enhanced by ganciclovir in an animal model of brain tumors.

To further show that *TK* gene transfer was responsible for the ganciclovir sensitivity and enhanced antitumor effect of hrR3, we treated 10 rats with RH105, the HSV mutant that possesses a defective *TK* gene (8). Seven days later, these animals received an i.p. injection of ganciclovir for 7 days. Fig. 1*C* shows that there were no long-term survivors in this group. This suggested that expression of *TK* in tumor cells was likely responsible for the antitumor effect of ganciclovir.

Histopathological Analysis. In a separate series of experiments, brains from rats that had received hrR3 plus saline (Fig. 2*A*) or hrR3 plus ganciclovir (Fig. 2*B*) were histopathologically analyzed at the end of drug treatment. Brains from the former group ($n = 14$) displayed large tumors in almost all cases, whereas no tumors or very small tumors were present in the latter group ($n = 14$). No tumor could be found in the brain of 1 of 14 animals from the hrR3 plus saline group and in 7 of 14 animals from the hrR3 plus ganciclovir group. Large areas of cavitary necrosis were present in brains from both groups. No cytotoxic effects to normal brain cells were present in either group. These results further confirm the antitumor action of the hrR3 vector and the potentiation of this action by ganciclovir.

***TK* Gene Transfer.** To show HSV vector-mediated transfer of the *TK* gene within the neoplasm, brains from animals harboring intracerebral 9L tumors were inoculated with hrR3 and then harvested 2 days later. A polyclonal antibody to HSV-*TK* (obtained from Dr. W. Summers, Yale University School of Medicine) was used to determine the presence of the antigen within tumor cells. The same sections were also double-stained for the *lacZ* gene product, whose expression in hrR3 is driven by the ICP6 immediate-early promoter (5). Fig. 3*A* shows the presence of numerous foci within the tumor of *lacZ*-positive "blue" cells admixed with *TK*-positive "brown" cells, indicative of active replication of hrR3. There were no *lacZ*-positive or *TK*-positive cells outside the tumor. Numerous tumor cells appear to coexpress both *lacZ* and *TK* gene products and, in several instances, endothelial cells in areas of tumor neovascularization showed both *lacZ* and *TK* antigen expression (Fig. 3*B*). Control tumors inoculated with RH105 did not display *TK* positivity (results not shown). These findings indicate that hrR3 mediated the relatively selective transfer of the *TK* gene within the mass of the neoplasm.

Discussion

The results presented in this study show that HSV vectors defective in ribonucleotide reductase are cytotoxic to tumor cells and confer ganciclovir susceptibility which can lead to complete brain tumor regression in a syngeneic experimental brain tumor model. This is likely a direct result of the HSV vector's ability to transfer the *TK* gene into the tumor as well as of its ability to selectively replicate and spread in brain tumor cells. The multiple antitumor capacity of HSV vectors (cytotoxicity and gene transfer) thus render them ideal candidates for gene therapy strategies against neoplasms. In contrast to other viral vectors, derived from retrovirus, adenovirus, and adeno-associated virus, hrR3 retains the ability to replicate in dividing cells due to complementation of its defective *RR* gene by mammalian enzymes with analogous function (5).

Although the mechanisms involved in tumor regression remain to be elucidated, it is likely that *TK* gene transfer in infected tumor cells results in the phosphorylation of ganciclovir to toxic nucleotide precursors, resulting in the death of cells expressing this gene as well as of neighboring uninfected tumor cells through the bystander effect (6, 10–13). This was evident in the cell culture experiments where ganciclovir potentiated the antitumor effect of hrR3, the HSV vector that retained an intact *TK* gene, but not that of RH105, the HSV vector with a disrupted *TK* gene. It is also likely that the drug also inhibits further HSV vector propagation due to its antiviral action (14). As discussed below, the use of chemosensitivity genes in HSV vectors is a useful strategy not only to enhance the antitumor action of HSV, but also to provide a means to abort further replication and spread of the virus mutant.

The other potential mechanism of tumor regression relies on the presence of an immune response against tumors (10, 11, 13). We hypothesize that the presence of HSV antigens within the tumor might contribute to the antitumor effect seen in the above study. They might also curtail further spread of the HSV vectors. In fact, lymphocytic infiltrates are present throughout tumors treated with HSV vectors.[4] A difference in the type or strength of immune response against RH105 compared to hrR3 might explain why the former vector was not as effective in inducing long-term animal survival (Fig. 1*C*; Ref. 4). To further support the role of an immune response, preliminary experiments have shown that surviving rats, previously treated with hrR3, become immune to additional intracerebral challenges with 9L cells.[5]

Histopathological analyses confirmed the antitumor effect of hrR3. Ganciclovir treatment resulted in complete destruction of tumors in a relatively large proportion of the brains analyzed. There was little evidence of ill effects to adjacent normal brain parenchyma, suggesting that formation of toxic nucleotides and the presence of a replication-compromised HSV vector were tolerated by treated animals. Surviving animals did not present evidence of widespread viral infection outside the central nervous system, as evidenced by their behavior as well as by their weight gain over the 90-day period. Preferential

[4] E. A. Chiocca and N. W. Kowall, unpublished observations.
[5] E. J. Boviatsis and E. A. Chiocca, unpublished observations.

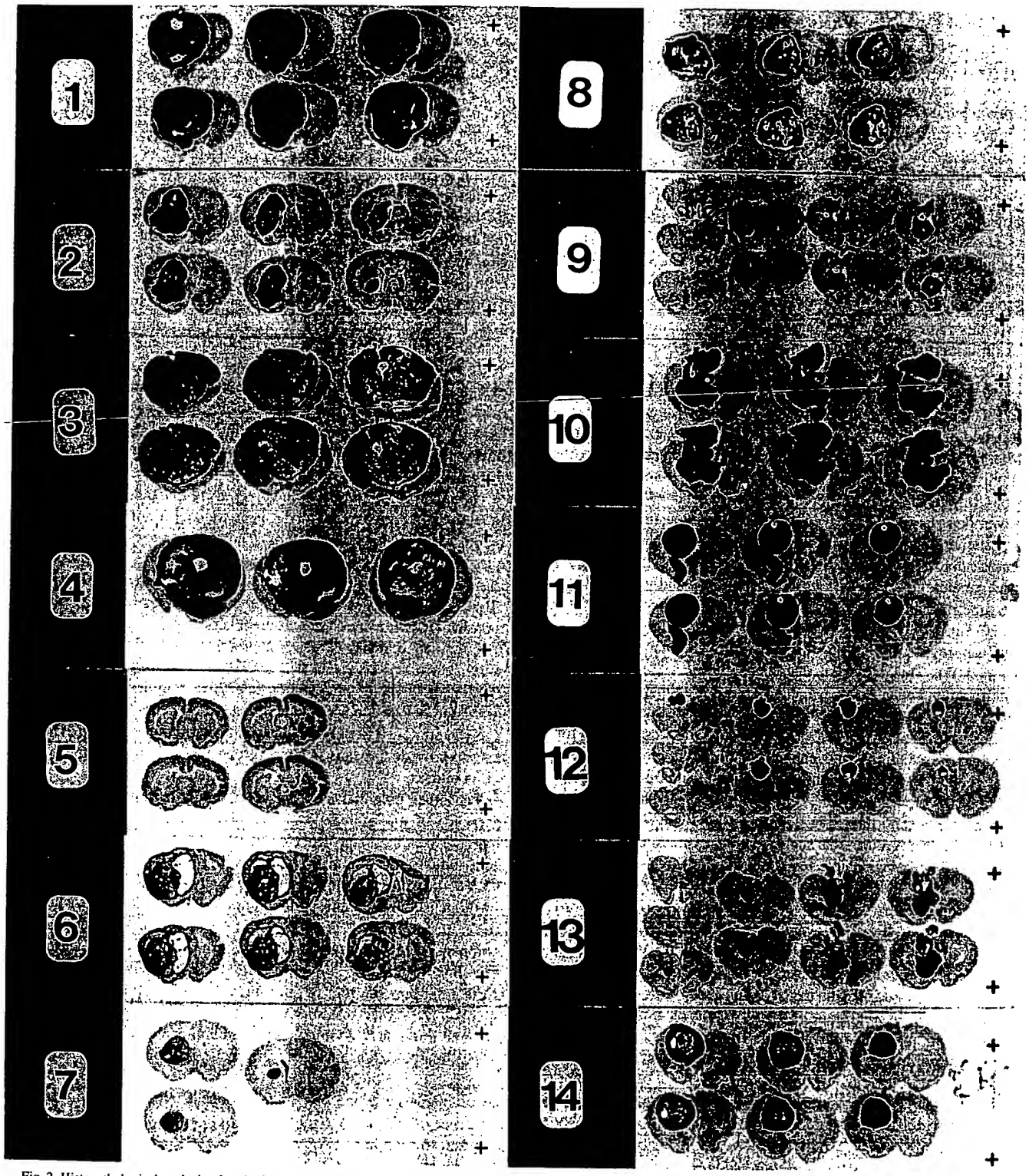


Fig. 2. Histopathological analysis of rat brains. Animals were sacrificed immediately after treatment with hrR3 plus saline (n = 14; A) or hrR3 plus ganciclovir (n = 14; B). Each histological slide contains 3 to 9 representative coronal sections (40 μm) from an animal's brain area where the 9L gliosarcoma cells had been implanted. Each slide thus contains sections from one animal's brain and the left portion of each slide has been marked by the labels "I" (representing animal 1), "2" (representing animal 2) and so forth, for both the hrR3 plus saline group (A) and the hrR3 plus ganciclovir group (B). Tumors are clearly visible because they stain darker with cresyl violet compared to adjacent normal brain areas. The light areas within each tumor represent areas of necrosis. × 1.

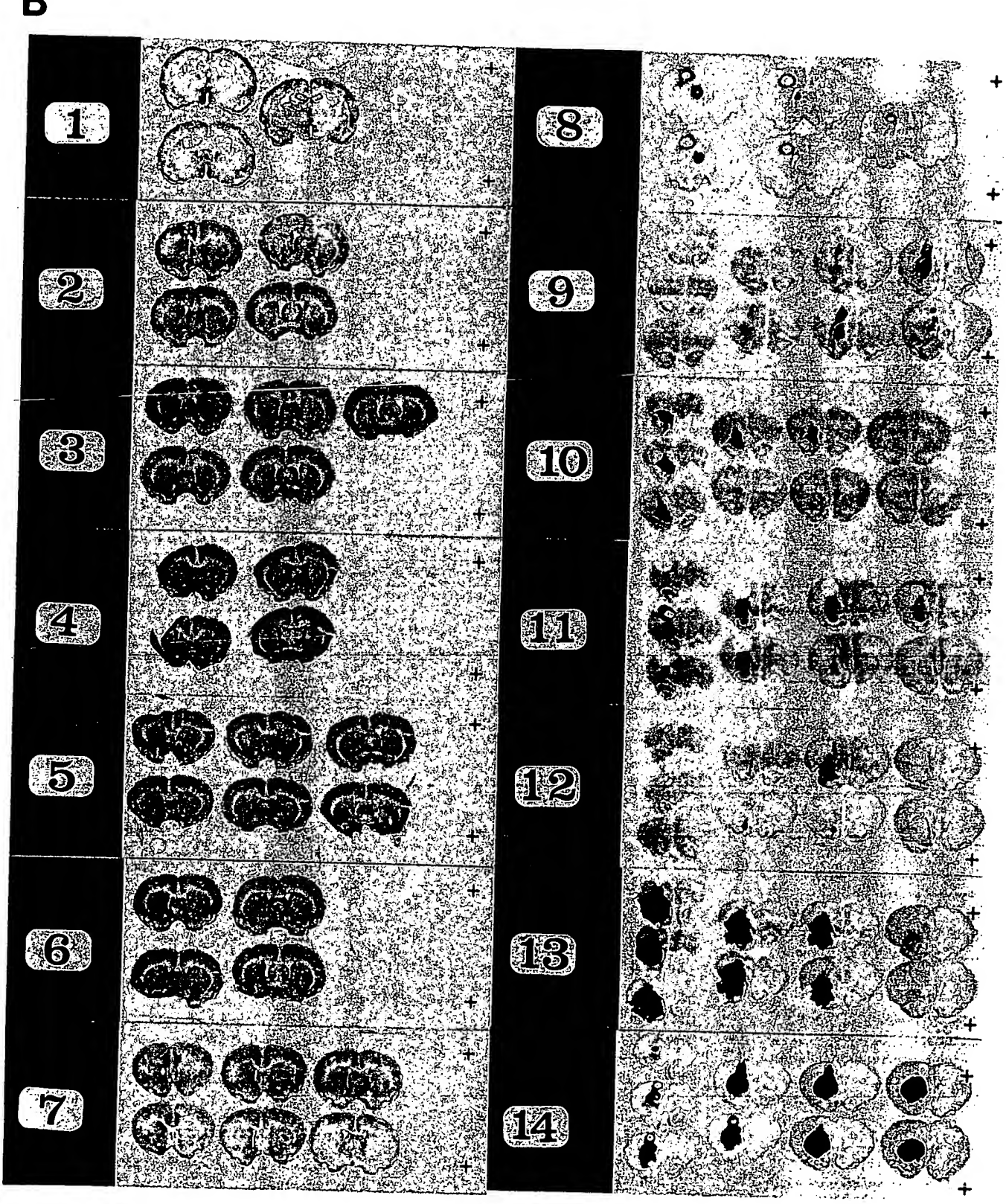
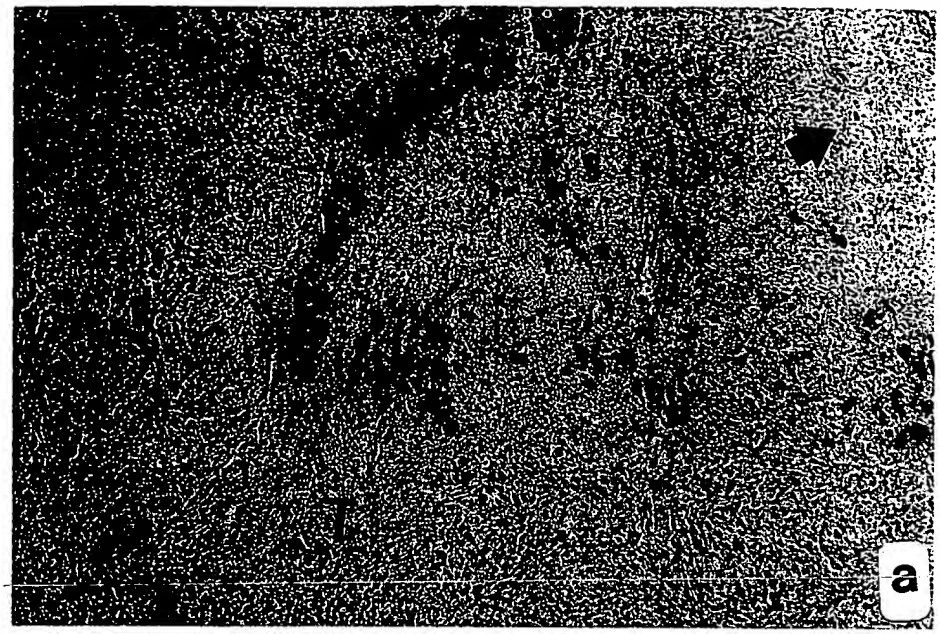


Fig. 2 *Continued*



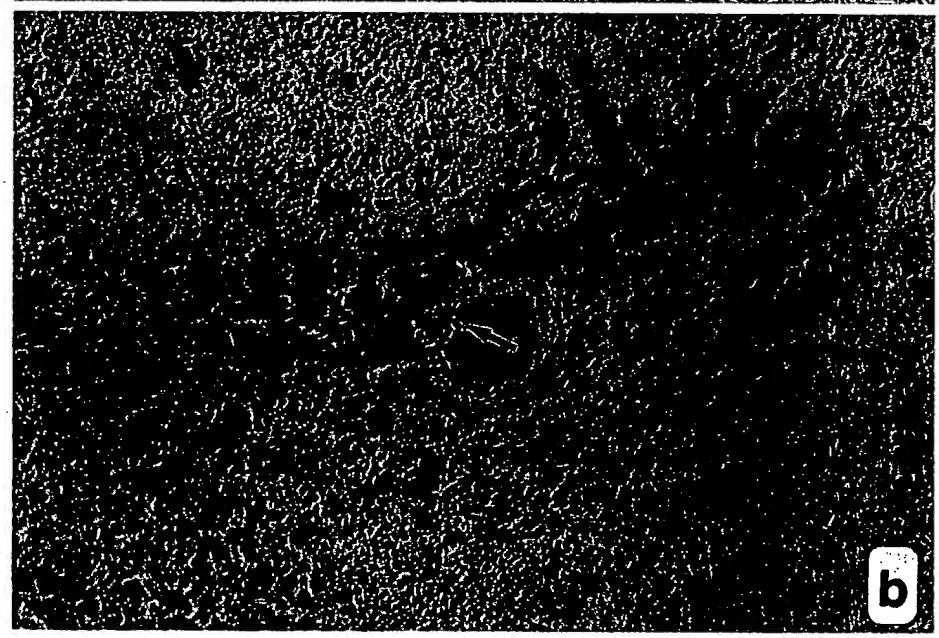


Fig. 3. *LacZ* enzyme histochemical and *TK* immunocytochemical assays. Coronal sections from rat brains with 9L tumors inoculated with hrR3 were double-stained first for *lacZ* gene expression as described (1, 4) and then for *TK* antigen expression using a polyclonal antibody raised against HSV-*TK* (a gift of Dr. William Summers, Yale). The "blue" cells within the tumors represent *lacZ*-positive cells, while the "dark brown" cells represent *TK*-positive cells. In (*a*), an area of brain tumor (indicated by *T*) is shown next to an area of normal brain parenchyma (*dark arrow*). Magnification = 4X. In (*b*), tumor cells are surrounding a blood vessel. Endothelial cells (*arrow*) also are *LacZ*- and *TK*-positive. × 10.

expression of the *lacZ* reporter gene within tumors compared to brain parenchyma was in agreement with previous findings (1, 4), providing a basis for the lack of histologically visible ill effects on endogenous neural cells. The immunocytochemical finding of TK positivity within tumor cells also provided a basis for the additional therapeutic boost provided by ganciclovir. The large genome of HSV is ideal for extensive genetic manipulations, and it has been estimated that up to 30 kilobases of the viral genome can be replaced with transgenes (14). The HSV vector could be rendered less neurovirulent by deletion of appropriate genes, such as $\gamma_{34.5}$ (15, 16), and its antitumor action can be enhanced by placing additional chemosensitivity genes (17, 18), apoptosis genes (19), or immune-response enhancer genes (20). These modifications would create an HSV vector which retains cytotoxicity for tumor cells, becomes less neurovirulent, and possesses two or more drug-sensitivity genes (and/or other therapeutic genes), allowing for multiple prodrug treatment of neoplasms as well as for the termination of further HSV vector infection.

Acknowledgments

We thank D. Ho and E. Mocarski (Stanford) for providing the RH105 mutant and S. Weller (University of Connecticut) for providing the hrR3 mutant. The generous gift of HSV-*TK* antibody by Dr. W. Summers (Yale) is also recognized.

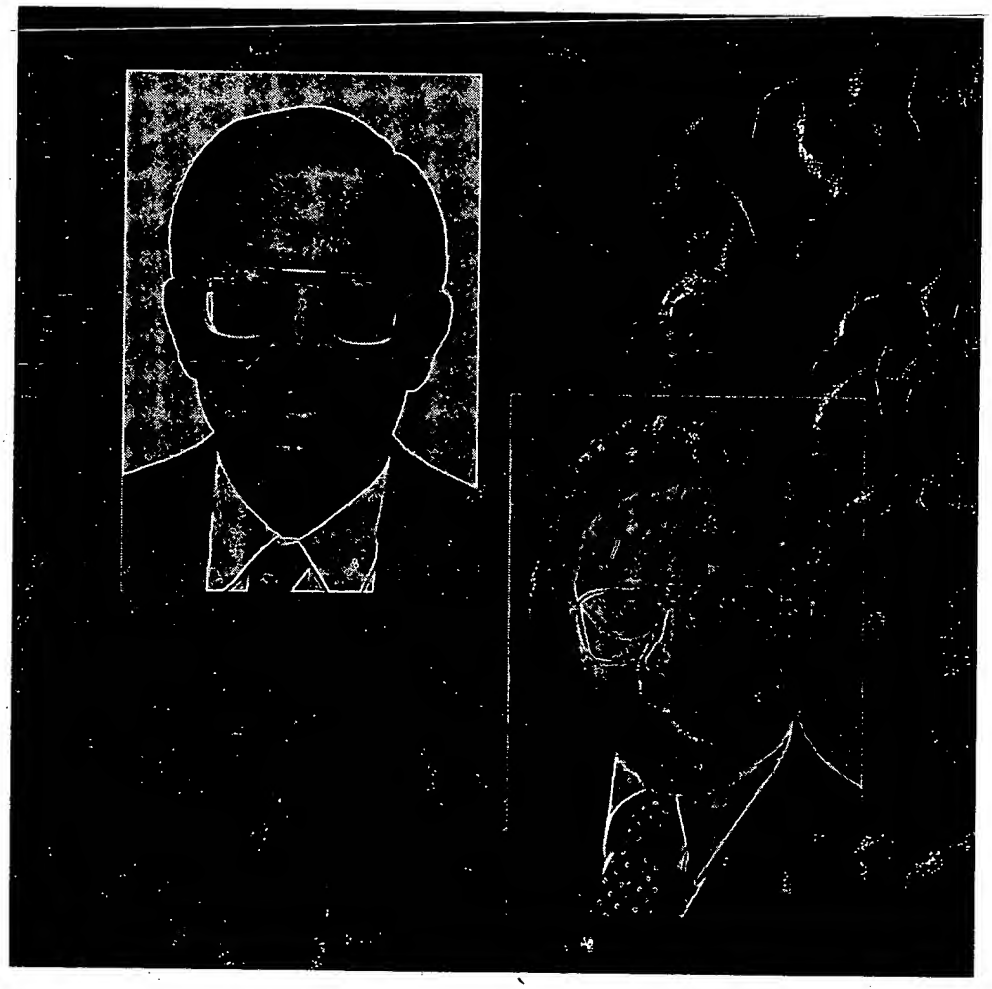
References

1. Boviatsis, E. J., Chase, M., Wei, M. X., Tamiya, T., Hurford, R. K., Jr., Kowall, N. W., Tepper, R. I., Breakefield, X. O., and Chiocca, E. A. Gene transfer into experimental brain tumors mediated by adenovirus, herpes simplex virus (HSV), and retrovirus vectors. Hum. Gene Therapy, *5:* 183–191, 1994.
2. Coen, D. M., Kosz-Vnenchak, M., Jacobson, J. G., Leib, D. A., Bogard, C. L., Schaffer, P. A., Tyler, K. L., and Knipe, D. M. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proc. Natl. Acad. Sci. USA, *86:* 4736–4740, 1989.
3. Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. I., and Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science (Washington DC), *252:* 854–856, 1991.
4. Boviatsis, E. J., Scharf, J. M., Chase, M., Harrington, K., Breakefield, X. O., and Chiocca, E. A. Antitumor activity and reporter gene transfer into rat brain neoplasms

inoculated with herpes simplex virus vectors defective in thymidine kinase or ribonucleotide reductase. Gene Therapy, *1:* 323–331, 1994.
5. Goldstein, D. J., and Weller, S. K. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. J. Virol., *62:* 196–205, 1988.
6. Moolten, F. L., and Wells, J. M. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. J. Natl. Cancer Inst., *82:* 297–300, 1990.
7. Weizsaecker, M., Deen, D. F., Rosenblum, M. L., Hoshino, T., Gutin, P. H., and Barker, M. The 9L rat brain tumor: description and application of an animal model. J. Neurol., *224* 183–192, 1981.
8. Ho, D. Y., and Mocarski, E. S. β-galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. Virology, *167:* 279–283, 1988.
9. Kaplan, E. L., and Meier, P. Nonparametric estimation for incomplete observation. J. Am. Stat. Assoc., *53:* 457–481, 1958.
10. Takamiya, Y., Short, M. P., Moolten, F. L., Fleet, C., Mineta, R., Breakefield, X. O., and Martuza, R. L. An experimental model of retrovirus gene therapy for malignant brain tumors. J. Neurosurg., *79:* 104–110, 1993.
11. Takamiya, Y., Short, M. P., Ezzeddine, Z. D., Moolten, F. L., Breakefield, X. O., and Martuza, R. L. Gene therapy of malignant brain tumors: a rat glioma line bearing the herpes simplex virus type 1-thymidine kinase gene and wild type retrovirus kills other tumor cells. J. Neurosci. Res., *33:* 493–503, 1992.
12. Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., and Abraham, G. N. The " bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res., *53:* 5274–5283, 1993.
13. Barba, D., Hardin, J., Sadelain, M., and Gage, T. H. Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. Proc. Natl. Acad. Sci. USA, *91:* 4348–4352, 1994.
14. Breakefield, X. O., and DeLuca, N. A. Herpes simplex virus for gene delivery to neurons. New Biol., *3:* 203–218, 1991.
15. Chou, J., and Roizman, B. The $\gamma_{34.5}$ gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. Proc. Natl. Acad. Sci. USA, *89:* 3266–3270, 1992.
16. Markert, J. M., Malick, A., Coen, D. M., and Martuza, R. L. Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. Neurosurgery, *32:* 597–602, 1993.
17. Wei, M. X., Tamiya, T., Chase, M., Boviatsis, E. J., Chang, T. K. H., Hochberg, F. H., Kowall, N. W., Waxman, D. J., Breakefield, X. O., and Chiocca, E. A. Experimental tumor therapy in mice with the cyclophosphamide-activating cytochrome P450 2B1 gene. Hum. Gene Therapy, *5:* 969–978, 1994.
18. Mroz, P. J., and Moolten, F. L. Retrovirally transduced *E. coli gpt* genes combine selectability with chemosensitivity capable of mediating tumor eradication. Hum. Gene Therapy, *4:* 589–595, 1993.
19. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Zhang, W. W., Owen-Schaub, L. B., and Roth, J. A. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type *p53* gene. Cancer Res., *54:* 2287–2291, 1994.
20. Yu, J. S., Wei, M. X., Chiocca, E. A., Martuza, R. L., and Tepper, R. I. Treatment of glioma by engineered interleukin 4-secreting cells. Cancer Res., *53:* 3125–3128, 1993.

Cancer Research

AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH



November 15, 1994
Volume 54 • Number 22
PP. 5745–6015
ISSN 0008-5472 • CNREA 8

Gene Therapy (1995) 2, 531–538

Transgene inheritance and retroviral infection contribute to the efficiency of gene expression in solid tumors inoculated with retroviral vector producer cells

T Tamiya[1,2], MX Wei[2], M Chase[2], Y Ono[1,2], F Lee[2], XO Breakefield[2,3] and EA Chiocca[1,2]

[1]Neurosurgery Service, Department of Surgery and [2]Molecular Neurogenetics Unit, Department of Neurology, Massachusetts General Hospital and [3]Program in Neurosciences, Harvard Medical School, Boston, MA, USA

One strategy to achieve efficient gene delivery into brain tumors employs the stereotactic implantation of fibroblasts that express a foreign gene and produce a retroviral vector bearing that gene. Another method involves the grafting of fibroblasts genetically engineered to produce a foreign gene product of interest. It is not clear to what extent retrovirus production in vivo provides an advantage over the grafting of genetically engineered cells for the purpose of achieving transgene expression. These two methods of gene delivery were compared in vivo by using the following cell lines: CRIP-MFG-LacZ cells, which express the lacZ gene and produce retrovirus vectors that bear this gene, and CRIP-LacZ cells, which express the lacZ gene, but do not produce retrovirus. Gene delivery was assessed in C6 gliomas established in the right frontal lobe of athymic mice. CRIP-MFG-LacZ or CRIP-LacZ cells were inoculated stereotactically into these tumors. When CRIP-MFG-LacZ cells were used, a relatively elevated level of lacZ gene expression was present in cells scattered throughout the tumor. Using a computerized imaging system, this expression occurred in approximately 10% of the tumor area at 1 week, 42% at 2 weeks, and 32% at 3 weeks. In contrast, with CRIP-LacZ cells, lacZ gene expression was much weaker and occurred in a more focal area within the tumor. This expression occupied approximately 5% of the tumor area at 1 and 2 weeks and had almost disappeared at 3 weeks. In both cases there was no notable expression of the transgene in normal brain cells. In conclusion, transgene expression in brain tumors was achieved in more cells, at higher levels, and for longer time periods with retroviral vector-producing cells than with genetically engineered fibroblasts. This efficiency of gene delivery likely results from direct in situ delivery of the transgene to tumor cells with subsequent inheritance of the reporter gene to progeny tumor cells.

Keywords: gene transfer; gene therapy; retrovirus vectors; brain tumors; cancer

Introduction

Several genes with therapeutic potential have shown effectiveness against experimental brain tumors. These genes encode prodrug-activating enzymes, such as HSV-TK[1–3] and the mammalian cytochrome P450 2B1 gene,[4] or immune-response enhancers, such as interleukin-4,[5] and antisense insulin growth factor-1.[6] Prodrug-activating genes have been delivered into brain tumor cells *in vivo*, by stereotactic grafting of the murine fibroblast cells that express the gene as well as producing the retrovirus vector bearing the transgene.[7–9] Immune-response enhancer genes have been delivered by mixing tumor cells with cells genetically engineered to express the transgene before implantation in animals.[5,6] It is not clear from these studies to what extent transgene delivery mediated by retrovirus vectors can augment the expression of a transgene in a solid tumor inoculated with genetically engineered cells. In this report, we show that the grafting of retrovirus vector producer cells enhances transgene expression in a neoplastic mass compared with the grafting of genetically engineered cells. The higher efficiency achieved by the *in situ* retrovirus method is due to transgene expression occurring not only in the genetically engineered packaging cells, but also in infected tumor cells and their clonal progeny. This finding has profound implications for the design of gene therapy clinical trials in humans afflicted with brain tumors.

Correspondence: Dr EA Chiocca, Molecular Neurogenetics Unit, Sixth Floor, Massachusetts General Hospital-East Building, Charlestown, MA 02129, USA

Received 18 November 1994; accepted 1 May 1995

Results

In vitro characterization of cell lines

The murine fibroblast packaging cells (designated as CRIP cells) are derived from NIH-3T3 fibroblasts and are designed to generate replication-defective, amphotropic retrovirus vectors,[10] such as MFG-*LacZ*.[11] To generate a comparable cell line that expresses the *lacZ* gene but does not produce retrovirus vector, we used the pLTRZ1 plasmid which contains the *lacZ* gene driven by the retroviral long terminal repeat (LTR) promoter without other retroviral sequences

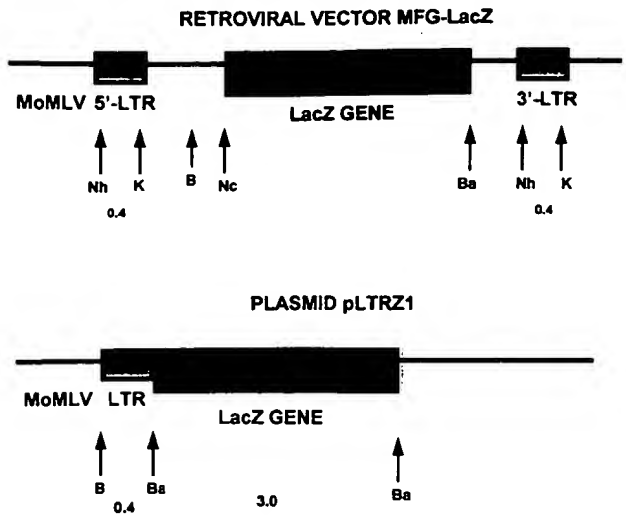


Figure 1 Schematic diagram of plasmid DNAs employed in the construction of CRIP-MFG-LacZ and CRIP-LacZ cells. The MFG-LacZ retroviral vector,[11] and the pLTRZ1 plasmid,[12] were employed for stable transfection into CRIP fibroblasts. The former procedure thus ends upon creating CRIP fibroblasts that generate a lacZ retrovirus as well as express lacZ. The latter generates CRIP fibroblasts that only express lacZ but do not generate retrovirus. Arrows represent restriction sites. Nh, NheI; K, KpnI; B, BglII; Nc, NcoI; Ba, BamHI; MoMLV, Moloney murine leukemia retrovirus; LTR, long terminal repeat. The values represent the approximate distance expressed in kilobases

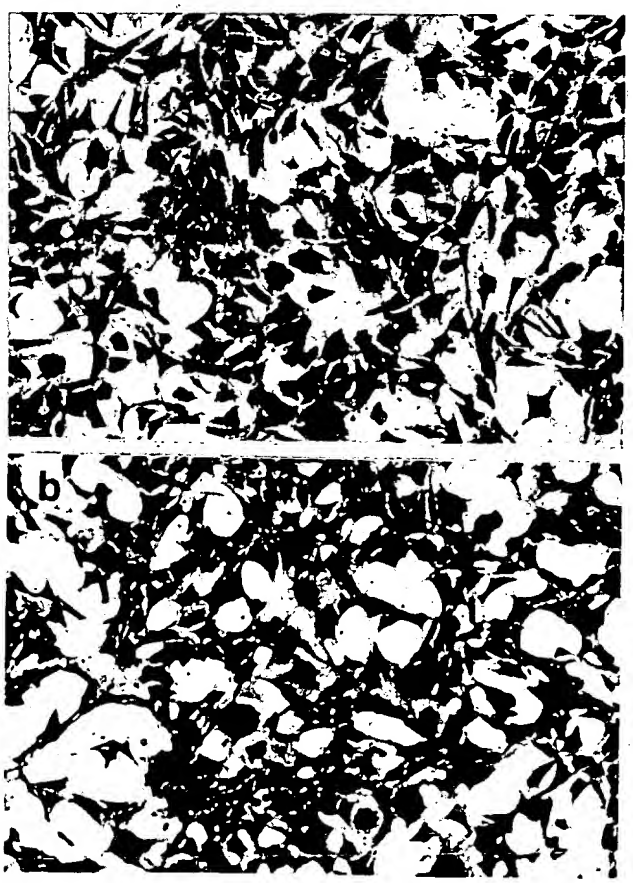


Figure 2 (a) LacZ-positive CRIP-MFG-LacZ and (b) CRIP-LacZ fibroblasts. Cultured cells were stained for lacZ gene expression with X-gal, as described.[3] Original magnification ×10

(Figure 1).[12] This plasmid was cotransfected into CRIP cells with a plasmid that encodes the neomycin phosphotransferase gene and, after selection in G418, a *lacZ* positive clone was selected and designated as CRIP-*LacZ*. CRIP-MFG-*LacZ* cells grew faster in culture than CRIP-*LacZ* cells (the average doubling time for the former was 13.7 h, while for the latter it was 20 h). The extent of *lacZ* gene expression was relatively similar for these two cell clones in culture (Figure 2a and b). To ensure that CRIP-MFG-*LacZ* cells produced a retrovirus vector while CRIP-*LacZ* cells did not, supernatants from each clone were harvested and added on to naive rat C6 glioma cells.[13] After 48 h, approximately 50% of C6 cells exposed to supernatant from CRIP-MFG-*LacZ* cells had become β-galactosidase positive, indicating that they had been infected with a retrovirus bearing the *lacZ* gene (Figure 3a). On the other hand, C6 cells exposed to supernatant from CRIP-*LacZ* cells had not acquired *lacZ*-positivity, indicating the absence of significant retroviral vector production (Figure 3b).

In vivo comparison between cell lines

To compare *lacZ* gene expression within experimental brain tumors grafted with CRIP-MFG-*LacZ* or CRIP-*LacZ* cells, C6 glioma cells were stereotactically implanted in the frontal lobes of athymic mice. Three

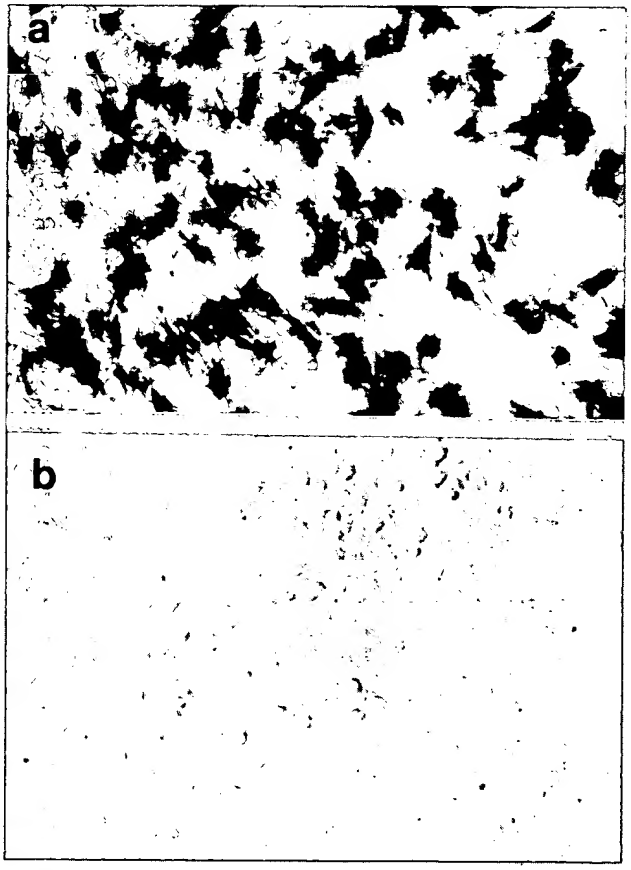


Figure 3 Retroviral infection of rat C6 glioma cells. Rat C6 glioma cells were exposed to supernatants harvested from CRIP-MFG-LacZ (a) or CRIP-LacZ (b) cells for 4 h. LacZ gene expression was then assayed 48 h later

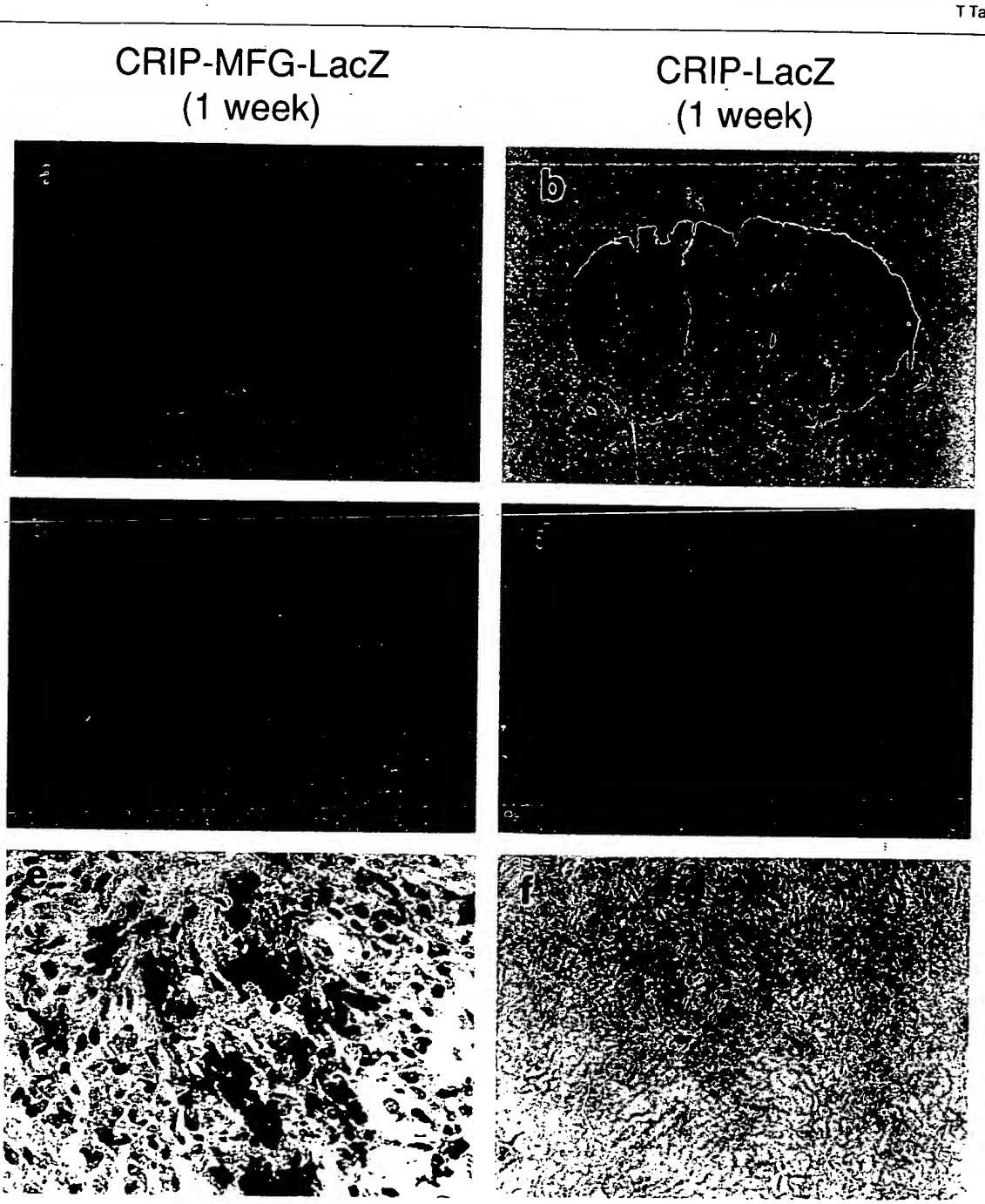


Figure 4 LacZ gene expression in C6 glioma tumors 1 week after injection of CRIP-MFG-LacZ or CRIP-LacZ cells. C6 glioma tumors were established in the frontal lobes of athymic mice. CRIP-MFG-LacZ (a, c and e) or CRIP-LacZ cells (b, d and f) were then inoculated stereotactically into the tumor. One week later, animals were killed and brain sections were stained with cresyl violet to identify tumors (a and b). Adjacent sections were also stained for lacZ gene expression (c and d). (e and f) High-power photomicrographs. (a–d) Original magnification ×1; (e and f) Original magnification ×40

days later, CRIP-MFG-*LacZ* or CRIP-*LacZ* cells were stereotactically inoculated into the same cerebral location. Animals were killed 1, 2 and 3 weeks later. Figure 4 shows representative sections of brains harvested at 1 week. Large tumors were present in brains of animals from both groups (Figure 4a and b). *LacZ*-positive cells could be found throughout the tumor in brains from both animal groups, as shown by the diffuse blue staining visible in the low-power photomicrographs in Figure 4c and d. When magnified, these blue areas in the tumor contained *lacZ* positive cells, as shown in Figure 4e and f. The level of *lacZ* gene expression in individual cells within the tumor was much higher in the CRIP-MFG-*LacZ* implanted animals compared with the CRIP-*LacZ* animals (compare Figure 4e and f).

More pronounced differences were visible in the brains of animals from the 2-week time point. Figure 5a and c show the strong and extensive *lacZ*-positivity within the tumor inoculated with CRIP-MFG-*LacZ* cells, while the tumor that had received CRIP-*LacZ* cells showed evidence of weakly *lacZ*-positive cells in

a more restricted area around the needle tract (Figure 5b and d). This indicated that presence of a retroviral vector produced a dramatic enhancement in the expression of a transgene within a tumor 7-14 days after injection of retrovirus-producer cells.

The above pattern did not change in the brains of animals from the 3-week time point. Strong expression of the *lacZ* gene was evident in the tumors receiving CRIP-MFG-*LacZ* cells (Figure 6a), while it was barely, if at all, detectable in those receiving

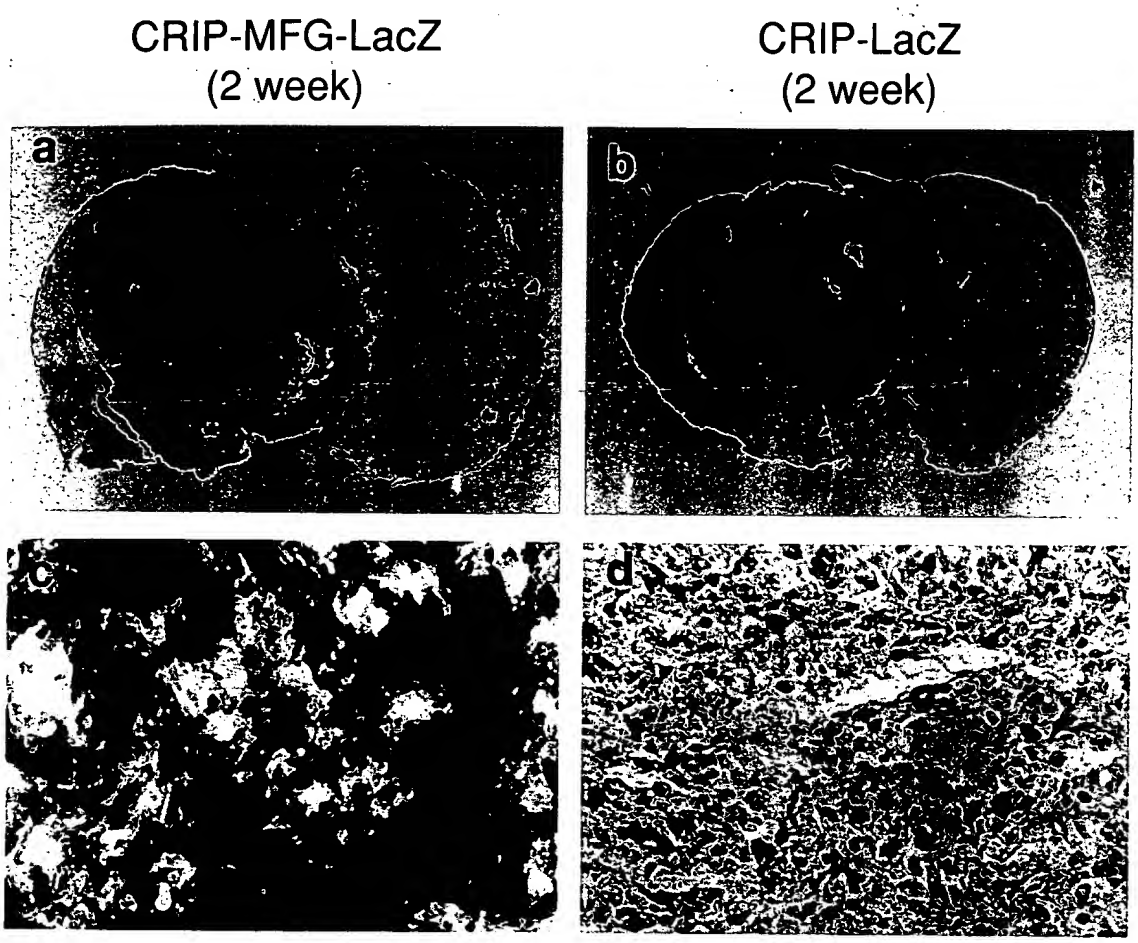


Figure 5 LacZ gene expression in C6 glioma tumors 2 weeks after injection of CRIP-MFG-LacZ or CRIP-LacZ cells. (a and b) Low-power photomicrographs: original magnification ×1; (c and d) high-power photomicrographs: original magnification ×40. Sections were stained with X-gal to detect lacZ-positive cells, followed by counterstaining with cresyl violet

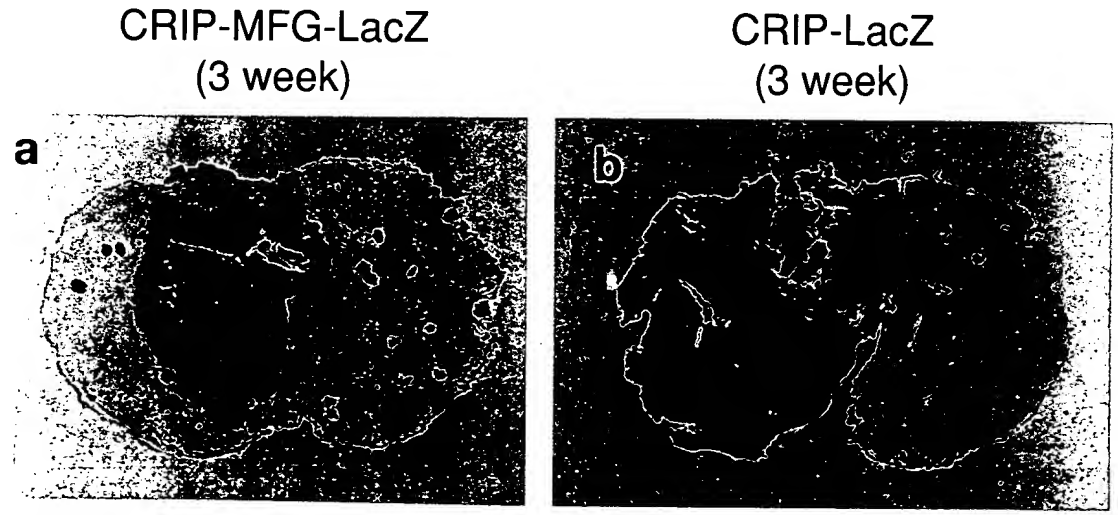


Figure 6 LacZ gene expression in C6 glioma tumors 3 weeks after injection of CRIP-MFG-LacZ or CRIP-LacZ cells. Sections were stained with X-gal, followed by counterstaining with cresyl violet

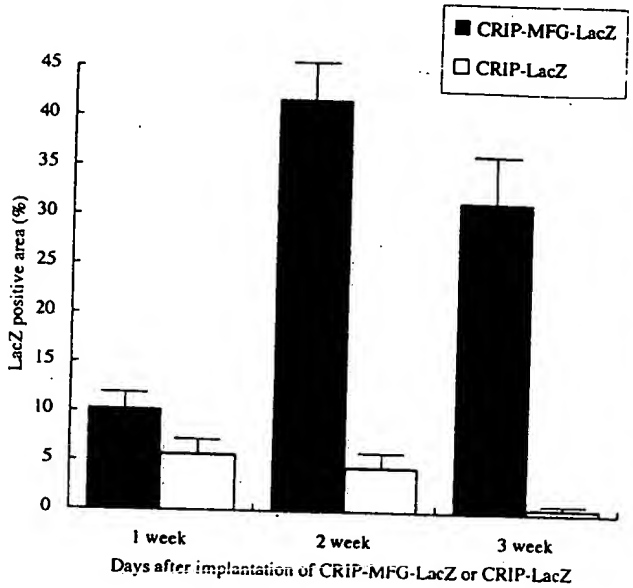


Figure 7 Quantitative analysis of lacZ-positive area in brain sections. Through computer-assisted image analysis, the percentage of lacZ-positive area within the tumor was measured for both CRIP-MFG-LacZ and CRIP-LacZ implants

CRIP-*LacZ* cells (Figure 6b). No β-galactosidase reactivity was seen in normal brain tissue surrounding the tumors.

Quantitative evaluation of lacZ gene expression

The anatomic density of *lacZ* gene expression was measured in the brains of animals from both groups by using computerized image analysis to calculate the total sum of *lacZ*-positive cellular areas in a section and express it as a percentage of the total tumor area in that section. Figure 7 reveals that, at one week, the mean percentage of *lacZ*-positive area within a tumor was about twice as large in the tumors treated with CRIP-MFG-*LacZ* compared with those treated with CRIP-*LacZ*. At the 2-week time point the mean *lacZ*-positive area within the tumor had increased about four-fold in the CRIP-MFG-*LacZ* group, to occupy approximately 42% of the total tumor area. In contrast, there was no increase in the *lacZ*-positive area in the CRIP-*LacZ* group, remaining at approximately 5%. By 3 weeks, there was a slight decrease in the average *lacZ*-positive tumor area for the CRIP-MFG-*LacZ* group to 33% ± 5% s.e.m., while the average *lacZ*-positive area diminished to about 1% in the CRIP-*LacZ* group.

In control experiments, CRIP-MFG-*LacZ* or CRIP-*LacZ* cells were implanted in the subcutaneous flank of athymic mice. There was essentially no significant growth of a tumor over a 3-week period (Figure 8a). CRIP-MFG-*LacZ* or CRIP-*LacZ* cells were also stereotactically implanted in the brains of athymic mice. Few *lacZ*-positive cells were visible 1 week later and none 3 weeks later (Figure 8b and c). These results indicate that the CRIP-MFG-*LacZ* and the CRIP-*LacZ* cells do not grow to form subcutaneous or intracerebral tumors in nude mice.

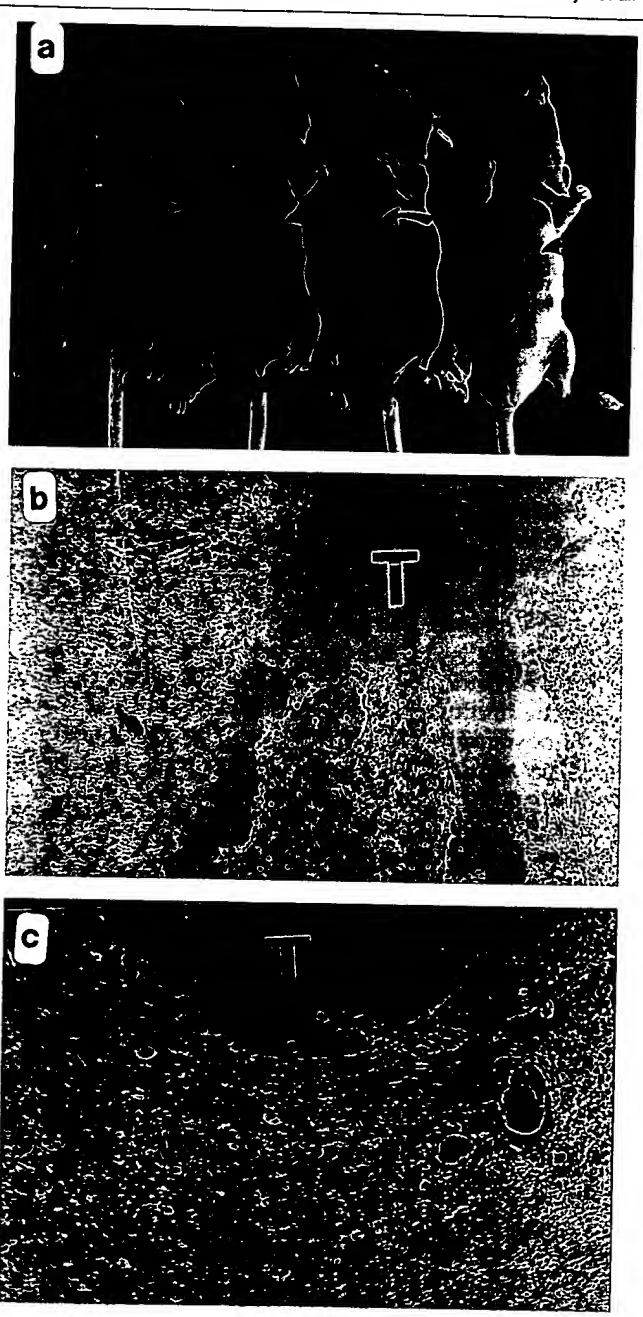


Figure 8 Lack of in vivo growth of CRIP-LacZ and CRIP-MFG-LacZ cells. (a) Subcutaneous injections of CRIP-LacZ or CRIP-MFG-LacZ cells failed to produce tumors at 3 weeks. The two mice on the left received a subcutaneous injection of CRIP-LacZ cells while the two mice on the right received an injection of CRIP-MFG-LacZ cells. The black arrows indicate the site of injection. (b) CRIP-LacZ cells were stereotactically inoculated into nude mice brains which were harvested 3 weeks later and stained for lacZ expression. The 'T' represents the needle tract. (c) Brains inoculated with CRIP-MFG-LacZ cells were also stained for lacZ expression. The 'T' represents the needle tract

Discussion

The findings of this report underscore two main aspects of the *in vivo* gene therapy for solid tumors in general and brain tumors in particular, namely the relatively limited anatomic and temporal fate of the injected packaging cells and the superior efficiency of retrovirus-mediated gene transfer compared with

gene expression mediated by genetically engineered cells. This superiority (manifested by transgene expression in more cells, at higher levels, and for a longer time period) is likely due to prolonged infection and clonal expansion of retrovirally infected tumor cells. In addition, there was a prolonged, strong expression from the LTR in tumor cells in contrast to the same promoter in packaging cells *in vivo*. This cannot be due to the site of integration, as it would be different in different tumor cells, and is assumed to be due to ongoing tumor cell division and availability of its genomic DNA to transcription factors.

The use of the CRIP-*LacZ* cells permits an evaluation of the anatomic distribution and temporal fate of the murine fibroblasts employed to package retrovirus vectors without the confounding variable of concomitant detection of retrovirally infected tumor cells. Our results show that throughout the course of the study the packaging cells do not migrate within the tumor. The effective life-span of the packaging cells also appears to be limited to 2 weeks, even in athymic animals. This is in general agreement with the findings from Short *et al* who showed by immunostaining for fibronectin, S-100, and glial fibrillary acidic protein (GFAP) that the producer cells do not survive more than one week after their grafting in a C6 glioma.[7] Similar results were reported in dexamethasone-treated rats by Ram *et al*.[14] Our results provide additional experimental evidence regarding the limited life-span of the retrovirus-producer cells. It could be argued that the decrease in transgene expression is associated with down-regulation of the *lacZ* gene in CRIP-*LacZ* (or CRIP-MFG-*LacZ*) cells. Either cause (promoter down-regulation or lack of producer cell survival) for the observed decrease in transgene expression ultimately would lead to an identical effect, that is the diminution of retrovirus vector production 1–2 weeks after producer cell grafting. The lack of intracerebral or subcutaneous growth by CRIP-MFG-*LacZ* or CRIP-*LacZ* cells provides additional evidence that the *lacZ*-positivity seen in tumors at the 2–3 week time points is associated with tumor cells and not with producer cells.

The most likely model that would explain the sum of these findings is that during the course of the first 2 weeks, there is an ongoing infection of tumor cells by retrovirus vectors. Subsequent clonal expansion and inheritance of the transgene would contribute to the large increase in reporter gene expression seen at the 2-week time point. It is possible that the initial injection of the retrovirus vector producer cells allows for some diffusion within the tumor mass. Even more likely is the possibility that the infected tumor cells and their progeny might migrate in the tumor which could explain the relatively wider anatomic distribution of the *lacZ* gene in the animals inoculated with CRIP-MFG-*LacZ* cells compared with those inoculated with CRIP-*LacZ* cells.

The concepts of therapeutic or reporter gene inheritance in tumors and of a window of time during which there is maximum transgene expression have profound implications for any clinical trial in humans, in which a prodrug-activating gene is retrovirally transduced in the brain tumors. For instance, a 1–2 week lag time is usually allowed for retroviral transduction of the HSV*tk* gene in solid tumor masses before administration of the prodrug, ganciclovir. To further optimize ganciclovir's anticancer effect, it would seem reasonable to know how long after producer cell injection HSV*tk* gene expression reaches an anatomical maximum, before instituting treatment with the prodrug. In this setting, the most favorable chemotherapeutic effect would result from treating the neoplastic mass that possesses the largest percentage of transgene-positive tumor cells. Obviously, in the tumor model we employed for this study, a maximum therapeutic effect might be expected 2–3 weeks after inoculation of the producer cells. It is likely that this might vary from tumor to tumor, depending on the percentage of cells in the S-phase of the cell cycle. In conclusion, our results lead us to believe that one possible way to improve a prodrug-activating gene therapy strategy might involve knowledge of the time in which maximum transgene expression is achieved in the treated neoplasm after grafting a producer cell line.

Materials and methods

Plasmids and cell cultures

The amphotropic retrovirus producer line, ψCRIP (derived from mouse 3T3 fibroblasts) was obtained from Dr Richard Mulligan (Massachusetts Institute of Technology). Plasmid pLTRZ1 was obtained from Dr Donald Coen (Harvard Medical School) and it contains the *lacZ* gene under the control of the Moloney murine leukemia virus LTR.[12] The CRIP-MFG-*LacZ* fibroblasts were obtained from Drs Glen Dranoff and Richard Mulligan (Massachusetts Institute of Technology). These produce retrovirus vectors bearing the *lacZ* gene at a titer equivalent to 4×10^5 colony-forming units (c.f.u.)/ml. To make a cell line that expresses the *lacZ* gene without producing a retrovirus vector encoding that gene, CRIP fibroblasts were cotransfected with pLTRZ1 and pRSV*neo*, a plasmid encoding for neomycin resistance. Several clones were selected for growth in the presence of G418 (1 mg/ml) and one clone was chosen by enzyme histochemical analysis for high expression of β-galactosidase.[15] All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% calf serum (CS) (GIBCO BRL, Gaithersburg, MD, USA) and 1% penicillin/streptomycin (PS, GIBCO) at 37°C in an atmosphere containing 5% carbon dioxide. Rat C6 glioma cells were cultured in the same medium containing 10% fetal calf serum.[13]

Culture studies

Rat C6 glioma cells were plated at a density of 2×10^5 cells per p100 dish (Corning Glass Works, Corning, NY, USA). Cells were incubated 24 h later, with

supernatants harvested from CRIP-MFG-*LacZ* or CRIP-*LacZ* cells and filtered through 0.45-μ pores (Gelman Sciences, Ann Arbor, MI, USA). Infections were carried out in the presence of 10 μg/ml of polybrene for 2 h. C6 cells were then washed with Hank's buffered saline (HBS) and reincubated in DMEM/FCS/PS. The C6 cells were washed twice, 48 h later, with HBS and fixed with 0.5% glutaraldehyde for 10 min. They were then incubated at 37°C for 24 h in a solution containing 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% NP40, and 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Sigma, St Louis, MO, USA), pH = 7.3.[15]

Animal studies

Animal studies were conducted according to the institutional guidelines promulgated by the Massachusetts General Hospital Committee on Animal Care. For animal injections, cultured cells were trypsinized and replated 24 h before injection into animals. At this time, they were harvested by trypsinization, washed in HBS, and concentrated in DMEM. Athymic mice were anesthetized with an intraperitoneal injection of ketamine (100 mg per kg of body weight) (Parke Davis, Morris Plains, NJ, USA) and xylazine (20 mg per kg of body weight) (Mobay, KS, USA). Surgical procedures were performed in a sterile fashion. After immobilizing the mice in a stereotactic apparatus (Kopf Instruments, Turingia, CA), a small incision was made in the skin overlying the skull and a small burr hole was drilled approximately 0.5 mm frontal and 0.5 mm right lateral to the bregma. Rat C6 glioma cells (10^3 cells in 2 μl) were stereotactically inoculated at a depth of 2.5 mm through the burr hole using a Hamilton syringe. The inoculation period was 5 min, with 2 min allowed for needle retraction. The same burr hole was employed 3 days later for the stereotactic grafting of 2×10^6 CRIP-MFG-*LacZ* or CRIP-*LacZ* cells in 25 μl of DMEM. These injections were carried out stereotactically over a period of 10 min with 5 min allowed for needle retraction. One, two and three weeks after grafting the CRIP-MFG-*LacZ* or CRIP-*LacZ* cells, three animals from each group were killed by intracardiac perfusion with 10 mM sodium phosphate buffer, pH = 7.3 in 0.9% sodium chloride (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Brains were placed in 4% PFA in PBS solution for 24 h at 4°C and then for an additional 24 h in 30% sucrose in 10 mM sodium phosphate buffer, pH = 7.3. Brains were sectioned on a sledge microtome (50-μ slices) and placed for 24 h at 37°C in the X-gal reagent. Sections were then rinsed in PBS, mounted on to slides and counterstained with cresyl violet. To provide an estimate of gene transfer, three sections from each brain (three brains per group) were randomly selected and their total tumor area as well as the *lacZ*-positive tumor areas were calculated by using computerized image analysis (OPTIMAS). Statistical analyses were performed using analysis of variance (ANOVA) with Fisher *post hoc* analysis on MacIntosh statistical software.

To evaluate the proliferation rate of CRIP-*LacZ* and of CRIP-MFG-*LacZ* cells, 4×10^4 cells were plated per dish and their numbers were determined by Coulter counting 1, 3, 5 and 7 days later.

To evaluate the *in vivo* growth characteristics of CRIP-*LacZ* and CRIP-MFG-*LacZ* cells, 2×10^6 cells were injected subcutaneously into the flanks of athymic mice in a volume of 200 μl. The volume of tumors was estimated on a weekly basis by tridimensional measurements with external calipers. To study the intracerebral growth of these cell lines, 2×10^6 CRIP-MFG-*LacZ* or CRIP-*LacZ* cells were stereotactically injected into the right frontal lobes in a volume of 25 μl. Animal brains were harvested 1, 2 and 3 weeks later and stained for β-galactosidase expression as described in the previous paragraphs.

Acknowledgements

Supported by grants from the National Institutes of Health NINDS NS 24279-08 (to EAC and XOB). MXW is a fellow of the Elsa Pardee Foundation.

References

1 Moolten FL. Tumor chemosensitivity conferred by inserted thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* 1986; **46**: 5276–5281.
2 Ezzeddine ZD *et al.* Selective killing of glioma cells in culture and *in vivo* by retrovirus transfer of the herpes simplex virus thymidine kinase gene. *New Biol* 1991; **3**: 608–614.
3 Boviatsis EJ *et al.* Long term survival of rats harboring brain neoplasms treated with ganciclovir and a herpes simplex virus vector that retains an intact thymidine kinase gene. *Cancer Res* 1994; **54**: 5745–5751.
4 Wei MX *et al.* Experimental tumor therapy in mice with the cyclophosphamide-activating cytochrome P450 2B1 gene. *Hum Gene Ther* 1994; **5**: 969–978.
5 Yu JS *et al.* Treatment of glioma by engineered interleukin4-secreting cells. *Cancer Res* 1993; **53**: 3125–3128.
6 Trojan J *et al.* Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. *Science* 1993; **259**: 94–97.
7 Short MP *et al.* Gene delivery to glioma cells in rat brain by grafting of a retrovirus packaging cell line. *J Neurosci Res* 1990; **27**: 427–433.
8 Takamiya Y *et al.* An experimental model of retrovirus gene therapy for malignant brain tumors. *J Neurosurg* 1993; **79**: 104–110.
9 Culver KW *et al.* *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 1992; **256**: 1550–1552.
10 Danos O, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA* 1988; **85**: 6460–6464.
11 Dranoff G *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and

long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA* 1993; **90**: 3539–3543.

12 Andersen JK, Garber DA, Meaney CA, Breakefield XO. Gene transfer into mammalian central nervous system using herpes virus vectors: extended expression of bacterial *lacZ* in neurons using the neuron-specific enolase promoter. *Hum Gene Ther* 1992; **3**: 487–499.

13 Benda P, Someda K, Messer J, Sweet WH. Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. *J Neurosurg* 1971; **34**: 310–323.

14 Ram Z *et al*. *In situ* retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res* 1993; **53**: 83–88.

15 Price J, Turner D, Cepko C. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc Natl Acad Sci USA* 1987; **84**: 156–160.

Volume 2 • Number 8 • October 1995

Therapy

STOCKTON